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NOTES

Using Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry To Detect Carbapenem Resistance within 1 to 2.5 Hours▽

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In recent years, the percentage of carbapenem-resistant bacteria has increased at an alarming pace and become a major threat for patient survival. Carbapenemase-induced carbapenem resistance can be confirmed through the detection of carbapenem degradation using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). This method works for strains carrying NDM-1, VIM-1, VIM-2, KPC-2, and different IMP enzymes.

Carbapenem resistance is the latest obstacle in the fight against Gram-negative bacterial infections (2). One underlying resistance mechanism is the production of carbapenemases (3). To date, more than 100 different enzymes have been described (4, 14). Most recently, Kumarasamy et al. described NDM-1 (8). Carbapenem resistance can be detected using susceptibility testing methods (e.g., MIC determination, agar diffusion, and modified Hodge Test) and DNA-based methods (i.e., PCR). However, the time to results is at least 18 h for susceptibility testing methods, and some enzymes do not show proper resistance *in vitro* (14). PCR results are available the same day but at high costs per reaction. Due to its specificity, PCR can only detect known enzymes (1). We developed a matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) protocol with a time to result of 1 to 2 1/2 h which can detect a broad range of enzymes. It is easy to perform and low in cost. MALDI-TOF MS is a method where material is ionized in a high-vacuum chamber and accelerated in an electric field. The fragment size can be inferred from the time of flight of the ionized fragments. In the last 2 years, MALDI-TOF MS has become a standard identification tool for bacteria, reducing the time to identification of bacteria to 1 workday (11). For susceptibility testing, MALDI-TOF MS is not yet an established method. However, in 2002, Du et al. described the possibility of differentiating between methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA) by utilizing the fact that the complete spectra differed greatly in a group of 76 different strains. Nevertheless, 7 of 76 results were mismatches compared to PCR results (*mecA* gene) (6). In 2007, Malakhova et al. described the possibility of detecting fluoroquinolone resistance in *Streptococcus pneumoniae* strains through detecting mutations in the fluoroquinolone target structures (9). Finally, in 2009, Marinach et al. described a minimal profile change concentration (MPCC) for *Candida albicans* strains and fluconazole, i.e., the lowest drug concentration at which a mass spectrum profile change can be detected. There was a 94% concordance with MIC results in terms of resistance in a group of 17 different strains (10). Here, we used MALDI-TOF MS to monitor the *in vitro* activity of carbapenemases, i.e., the degradation of the carbapenem into the hydrolyzed and decarboxylated product.

Bacterial strains were grown for 18 to 24 h on 5% sheep blood agar plates (Becton Dickinson, Germany) at 36°C. We added one 10-μl loopful of bacteria to 1 ml of 0.45% NaCl (Braun-Melsungen, Germany) with or without 0.5 g/liter ertapenem in the original 1.5-ml Eppendorf tubes. We incubated carbapenemase- and non-carbapenemase-carrying strains for up to 2.5 h at 36°C. After the incubation, all tubes were centrifuged for 90 s at 12,000 × *g*. One microliter of the clear supernatant was applied to each target spot and left to dry at room temperature. One microliter of matrix (HCCA [α-cyano-4-hydroxycinnamic acid], high-pressure liquid chromatography [HPLC] grade; Fluka, Germany) was added to each target spot. MALDI-TOF MS was performed with a Bruker Daltonics microflex (Bremen, Germany) using 96-spot polished-steel targets. The protocol used was BioTyper_FC.par (provided by the manufacturer, 0 to 10,000 Da, 17 to 24% laser intensity). For one spectrum, about 200 to 300 shots were summed up, resulting in an intensity (arbitrary units) of $\geq 10^4$ for at least one peak. The HCCA peak at 379 Da was used for calibration. The mass spectrum between 438 and 530 Da was analyzed using flex analysis 3.0 software (provided by the manufacturer). A result was interpreted as positive for carbapenemase production if the

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TABLE 1. Characterization of bacterial strains

Enzyme[a]	Species	No. of isolates	Modified Hodge test result	MIC or range (mg/liter)[d]			Time to degradation of ertapenem (h)
				Ertapenem	Imipenem	Meropenem	
Carbapenemases[b]							
KPC-2 (A/2f)	*Klebsiella pneumoniae*	24/25	Positive (25/25)	4–≥8	2–≥16	1–8	1.5
	Escherichia coli	1/25					
NDM-1 (B/[c])	*Klebsiella pneumoniae*	3/5	Negative (4/5)	4–≥8	1–≥16	4–≥16	1
	Escherichia coli	2/5	Positive (1/5)				
IMP-1, IMP-7, (B, 3a)	*Pseudomonas aeruginosa*	3/3	Negative (1/3) Positive (2/3)	≥8	≥16	≥16	1
IMP-2, (B, 3a)	*Pseudomonas aeruginosa*	1/1	Positive (1/1)	≥8	≥16	≥16	1.5
IMP-8, IMP-16 (B, 3a)	*Pseudomonas aeruginosa*	3/3	Negative (1/3) Positive (2/3)	≥8	≥16	≥16	2.5
VIM-1 (B, 3a)	*Klebsiella pneumonia*	3/6	Negative (1/6)	4–≥8	1–≥16	≤0.25–≥16	1.5
	Citrobacter freundii	1/6	Positive (5/6)				
	Escherichia coli	1/6					
	Klebsiella oxytoca	1/6					
VIM-2 (B, 3a)	*Klebsiella pneumoniae*	1/4	Positive (4/4)	4–≥8	1–≥16	4–≥16	2.5
	Pseudomonas aeruginosa	3/4					
ESBL[d]	*Escherichia coli*	3	Negative (3/3)	≤0.5	≤1	≤0.25	No degradation
	Klebsiella pneumoniae	8	Negative (8/8)	≤0.5	≤1	≤0.25	No degradation
K1[e]	*Klebsiella oxytoca*	6	Negative (6/6)	≤0.5	≤1	≤0.25	No degradation
None detected (control strains)[f]	*Escherichia coli*	4	Negative (4/4)	≤0.5	≤1	≤0.25	No degradation
	Klebsiella pneumoniae	2	Negative (2/2)	≤0.5	≤1	≤0.25	No degradation
	Pseudomonas aeruginosa	2	Negative (2/2)	≥8	≤1	≤0.25	No degradation
	Enterobacter cloacae	2	Negative (2/2)	≤0.5	≤1	≤0.25	No degradation
	Proteus mirabilis	3	Negative (3/3)	≤0.5	2–4	≤0.25	No degradation

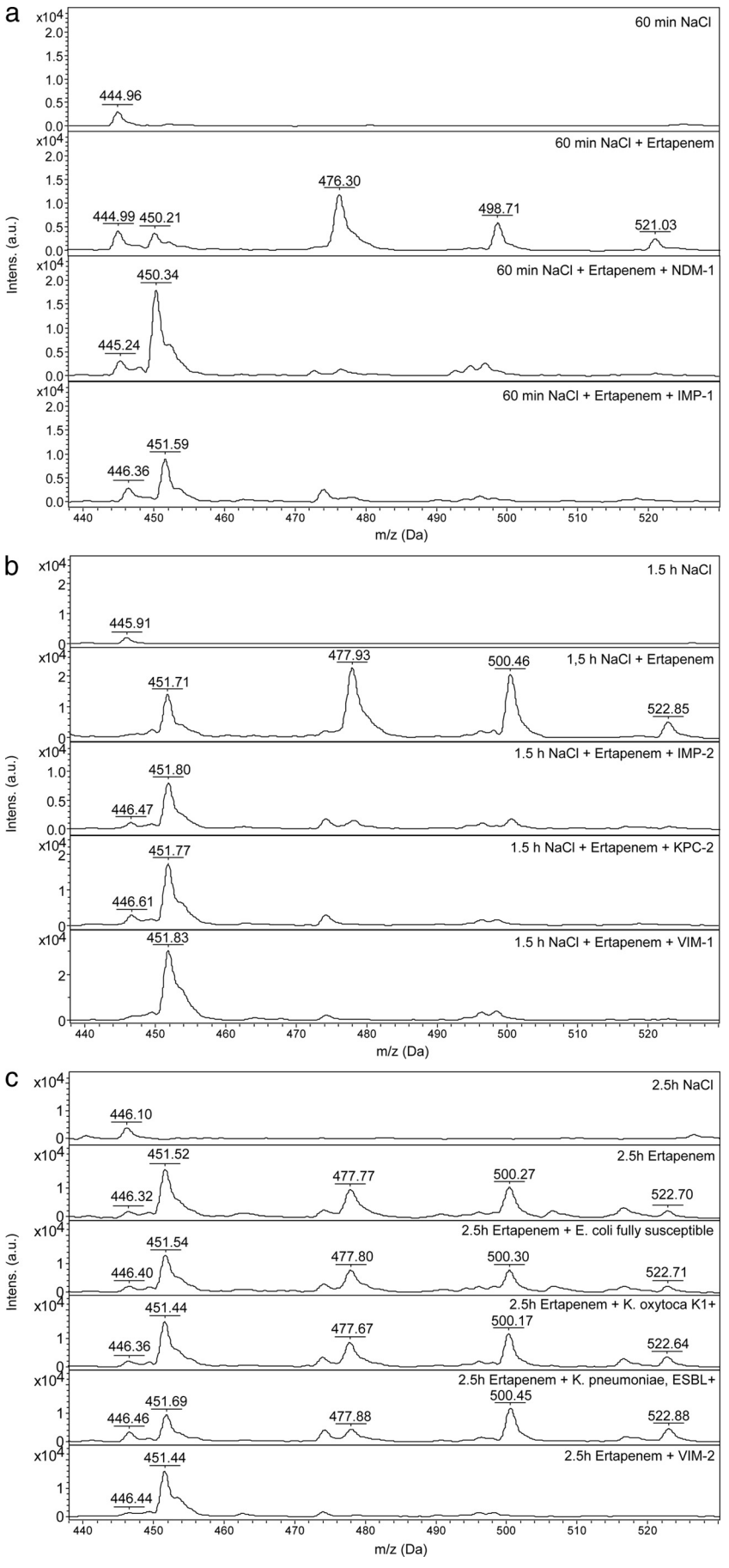
[a] Ambler/Bush-Jacoby classification (4, 16).
[b] Carriage of carbapenemase was confirmed by PCR (14) for NDM-1 using forward primer TCGATCCCAACGGTGATATT and reverse primer TGGATCAAG CAGGAGATCAA; product size, 287 bp (16).
[c] Not yet assigned.
[d] Carbapenem MICs and ESBL carriage were determined using the Vitek 2 Gram-negative resistance panels AST-N069 and AST-N118 (bioMérieux) or Etest (bioMérieux, AB Biodisk).
[e] Carriage of K1 in *Klebsiella oxytoca* was deduced from susceptibility test results using Vitek 2 Gram-negative resistance panels AST-N069 and AST-N118 as described previously (13).
[f] Susceptibility testing was done using Vitek 2 gram-negative resistance panels AST-N069 and AST-N118 (bioMerieux).

peaks for ertapenem (476, 498, and 521 Da) disappeared completely during the incubation time.

Initially, we established the characteristic mass spectrum of pure ertapenem. As expected, MALDI-TOF MS of ertapenem showed four peaks: 450 Da (hydrolyzed and decarboxylated ertapenem without sodium), 476 Da (ertapenem without sodium), 498 Da (monosodium salt), and 521 Da (disodium salt) (15). Subsequently, we conducted kinetic studies with strains known to carry carbapenemases (Table 1). We incubated them with ertapenem for up to 2.5 h. Strains carrying NDM-1 and IMP-1 needed just 1 h of incubation to completely degrade the added amount of ertapenem, i.e., until the peaks at 476, 498, and 521 Da vanished and only the peak at 450 Da was properly discernible. Strains carrying IMP-2, KPC-2, VIM-1, and VIM-2 needed 1.5, 1.5, 1.5, and 2.5 h, respectively (Fig. 1a, b, and c). The time to degradation correlated with the enzyme carried, not with the MICs of carbapenems. The spectrum of pure ertapenem was not altered by incubation at 36°C for up to 2.5 h (Fig. 1c). All spectra of supernatants of control strains showed peak profiles comparable to that of pure ertapenem (Fig. 1c). All spectra of supernatants of strains incubated without ertapenem (carbapenemase-carrying or non-carbapenemase-carrying strains) showed peak profiles comparable to that of pure NaCl (data not shown).

The described method of carbapenem resistance detection has many advantages compared to agar-based methods or PCR. First, depending on the type of carbapenemase, results can be available as soon as 1 h after the start of incubation. This is especially useful in an outbreak situation where the

FIG. 1. Ertapenem degradation is shown. (a) NDM-1-carrying *K. pneumoniae*, IMP-1-carrying *P. aeruginosa*; 60 min of incubation at 36°C; NaCl 0.45%; ertapenem concentration, 0.5 g/liter; *x* axis, mass per charge in Daltons [*m*/*z* (Da)]; *y* axis, intensity (arbitrary units [a.u.]). Data are representative of more than three independent experiments. (b) IMP-2-carrying *P. aeruginosa*, KPC-2-carrying *K. pneumoniae*, VIM-1-carrying *K. pneumoniae*; 1.5 h of incubation at 36°C; NaCl, 0.45%; ertapenem concentration, 0.5 g/liter; *x* axis, *m*/*z* (Da); *y* axis, intensity (a.u.). Data are representative of more than three independent experiments. (c) Control strains *E. coli* (fully susceptible), *Klebsiella oxytoca* ($K1^+$), *K. pneumoniae* (extended-spectrum β-lactamase positive [$ESBL^+$]), VIM-2-carrying *P. aeruginosa*; 2.5 h of incubation at 36°C; all reactions in 0.45% NaCl; ertapenem concentration, 0.5 g/liter; *x* axis, *m*/*z* (Da); *y* axis, intensity (a.u.). Data are representative of two independent experiments.


a
60 min NaCl
444.96
60 min NaCl + Ertapenem
476.30
444.99
450.21
498.71
521.03
60 min NaCl + Ertapenem + NDM-1
450.34
445.24
60 min NaCl + Ertapenem + IMP-1
451.59
446.36
Intens. (a.u.)
m/z (Da)
b
1.5 h NaCl
445.91
1,5 h NaCl + Ertapenem
477.93
500.46
451.71
522.85
1.5 h NaCl + Ertapenem + IMP-2
451.80
446.47
1.5 h NaCl + Ertapenem + KPC-2
451.77
446.61
1.5 h NaCl + Ertapenem + VIM-1
451.83
Intens. (a.u.)
m/z (Da)
c
2.5h NaCl
446.10
2.5h Ertapenem
451.52
477.77
500.27
446.32
522.70
2.5h Ertapenem + E. coli fully susceptible
451.54
477.80
500.30
446.40
522.71
2.5h Ertapenem + K. oxytoca K1+
451.44
477.67
500.17
446.36
522.64
2.5h Ertapenem + K. pneumoniae, ESBL+
451.69
500.45
446.46
477.88
522.88
2.5h Ertapenem + VIM-2
451.44
446.44
Intens. (a.u.)
m/z (Da)

carbapenemase is already identified. Second, this method is comparatively easy to perform. Only readily available reagents are used, and because MALDI-TOF MS is increasingly used in routine microbiology laboratories for the identification of bacterial strains, the hardware is already present in many laboratories. Third, the cost per determination is very low, less than one Euro per reaction. Finally, we want to stress the universality of the method. The principle of degradation product monitoring can be applied to other enzymatic resistance mechanisms.

Unfortunately, this method has a disadvantage. It cannot detect other carbapenem resistance mechanisms. These would include porin alterations and efflux mechanisms for *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* and porin alterations, efflux mechanisms, and PBP alterations for *Acinetobacter baumannii* (5, 7, 12).

We recommend this protocol as a fast method to confirm carbapenemase resistance in bacterial isolates.

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